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Report

Evidence for Extensive Recent Intron Transposition in Closely Related Fungi

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Summary

Though spliceosomal introns are a major structural component of most eukaryotic genes and intron density varies by more than three orders of magnitude among eukaryotes [1–3], the origins of introns are poorly understood, and only a few cases of unambiguous intron gain are known [4–8]. We utilized population genomic comparisons of three closely related fungi to identify crucial transitory phases of intron gain and loss. We found 74 intron positions showing intraspecific presence-absence polymorphisms (PAPs) for the entire intron. Population genetic analyses identified intron PAPs at different stages of fixation and showed that intron gain or loss was very recent. We found direct support for extensive intron transposition among unrelated genes. A substantial proportion of highly similar introns in the genome either were recently gained or showed a transient phase of intron PAP. We also identified an intron transfer among paralogous genes that created a new intron. Intron loss was due mainly to homologous recombination involving reverse-transcribed mRNA. The large number of intron positions in transient phases of either intron gain or loss shows that intron evolution is much faster than previously thought and provides an excellent model to study molecular mechanisms of intron gain.

Results and Discussion

Comparative Genomics Analyses Identify Extensive Recent Intron Gains

Intron density varies by more than three orders of magnitude among eukaryotes [1–3], but the causes of the large variation among species are controversial [3]. This stems from the lack of knowledge of proximate mechanisms leading to the insertion or loss of an intron within a gene. Analyses of eukaryotic genomes revealed that the ancestral eukaryote was intron rich [9], including the common ancestor of fungi and animals [10]. Thus, extensive ancestral intron loss and rare episodes of intron gain were suggested to account for the current variation in intron densities among eukaryotes [9, 11]. Rates of intron gains and losses (IGLs) establish an equilibrium intron density over evolutionary time. Rate variations and differences in effective population sizes are thought to influence intron densities among lineages [12]. The major mechanisms that were proposed to explain spliceosomal IGLs include reverse splicing, transposable elements, segmental genomic duplications, and nonsense-mediated decay [12–17]. However, direct evidence for the gain or loss of an intron at a particular location within a gene is scarce. IGLs must be associated with a transient phase of segregating alleles either carrying or lacking the intron within natural populations [18]. Until now, only 25 transient intraspecific intron presence-absence polymorphisms (PAPs) have been reported, one in *Drosophila teissieri* [8] and 24 in *Daphnia pulex* [5, 19]. In *Daphnia*, recently gained intron sequences were frequently associated with short repeats, suggesting a role for repair of double-strand breaks [5]. Furthermore, random genetic drift was shown to play a role in overcoming slightly deleterious effects of recent intron insertions.

We used genome-scale analyses of individuals from three closely related fungal species to identify the transient intron PAPs within species needed to elucidate mechanisms of IGL. *Mycosphaerella graminicola* is a globally distributed plant pathogenic fungus [20] that causes the most economically damaging foliar disease of wheat in Europe [21]. It has a high effective population size, regular recombination, and significant gene flow [22–24]. Several *M. graminicola* populations and a population sample of closely related species *Mycosphaerella* S1 and S2 were included to trace the evolution of IGLs. This multispecies population genomics framework allowed us to consider the influence of both recent speciation events and population structure on intron PAPs. We used the high-quality genome annotation data of the entirely assembled *M. graminicola* genome [25] as a reference for our analyses. We generated genome assemblies for seven further isolates and included two previously resequenced isolates of the same species [26]. Furthermore, we included genome assemblies of three related species: S1 (n = 5), S2 (n = 4), and *Septoria passerinii* (n = 1) as an outgroup species. Exon sequences surrounding intron positions were extracted for each of the 10,952 genes identified in *M. graminicola* and mapped to homologous positions in genome assemblies of all resequenced isolates and closely related species.

Transient Stages of Intron Gain and Loss Identified in Three Related Species

Within the clade composed of *M. graminicola* and its sister species S1, S2, and *S. passerinii*, we identified 219 fixed intron gains among species, representing approximately 1.2% of all introns identified in *M. graminicola* (Figure 1A). In addition to fixed intron gains, we identified substantial numbers of intron PAPs segregating within species. In total, we found 52, 20, and 2 intron PAPs in *M. graminicola*, S1, and S2, respectively. The 52 intron PAPs of *M. graminicola* were randomly distributed among 12 chromosomes and 37 genes (Figure 2A). Five genes displayed multiple intron PAPs. We used phylogenetic relationships among species to determine whether each intron PAP represented an intron gain or loss (see Table S1 available online). Among the 20 intron PAPs in S1, 14 showed transspecies polymorphism, with the same intron PAP present in S1 and *M. graminicola* (Table S2). Most of the reported intron PAPs likely had a recent origin, because a search for homologous positions in the outgroup species *S. passerinii* revealed only 11 homologous positions containing an intron. Reference genomes of more distantly related ascomycetes revealed only

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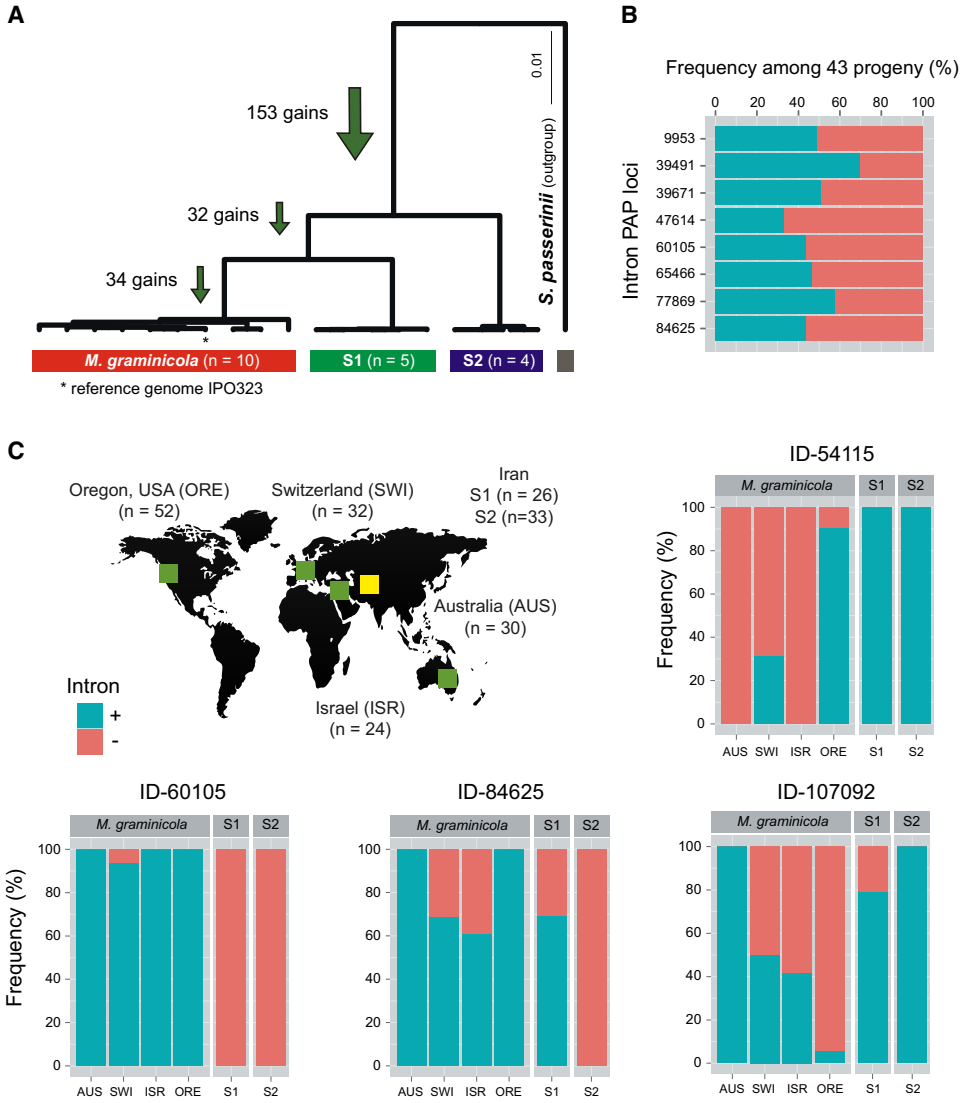

Figure 1. Comparative Genomics and Population Genetic Analyses of Intron Presence-Absence Polymorphisms

(A) Phylogenetic tree of the three closely related *Mycosphaerella* species and outgroup species *Septoria passerinii* based on an alignment of six conserved genes according to the standards of the fungal tree of life [31]. The number of genomes used in the screen and the identified intron gains among species are shown. The scale indicates number of substitutions per site.

(B) Segregation of eight intron presence-absence polymorphisms (PAPs) among 43 progeny. See Table S4 for detailed segregation scoring.

(C) Origin and sample size for four field populations of *M. graminicola* (green squares) and *Mycosphaerella* species S1 and S2 from Iran (yellow square). A total of 197 isolates were included. Intron PAP frequencies are shown for one locus with intron loss (ID-54115), another PAP with intron gain (ID-60105), and two intron PAPs showing transspecies polymorphism (ID-84625 and ID-107092). See Figure S1 for population genetic screens of seven additional intron PAP loci and Figure S3 for details on sequence polymorphism and neutrality tests on intron PAP loci.

a single homologous position (ID-41247) containing an intron (Table S3).

As a result of the large effective population size of plant pathogenic fungi, selection is expected to restrict intron gains to either neutral or beneficial insertions, and large, isolated populations may maintain transient stages during intron fixation. To understand population genetic processes affecting intron PAPs, we first crossed two *M. graminicola* isolates (ST99CH_1A5 and ST99CH_1E4) differing at 33 intron PAPs (Table S1). We found segregation for the eight analyzed intron PAPs among 43 offspring (Figure 1B; Table S4). We next assessed frequencies of 11 intron PAPs in four global populations of *M. graminicola* and a population of each S1 and S2 species (Figure 1C; Figure S1). Eight intron PAPs segregated within at least three populations. A shared polymorphism among distant populations indicates that the intron PAP was of recent origin and that both drift and selection were sufficiently weak to maintain variation. Fixation of either presence or absence of an intron was most often observed in the Australian population (8 out of 11 loci). This is consistent with earlier studies showing that *M. graminicola* was established in Australia through a recent founder event [27]. Similarly, in *Daphnia* a large proportion of recently gained introns were found in a genetically isolated population [5]. Population genetic processes such as gene flow, founder effects, and effective population size are likely to influence the outcome

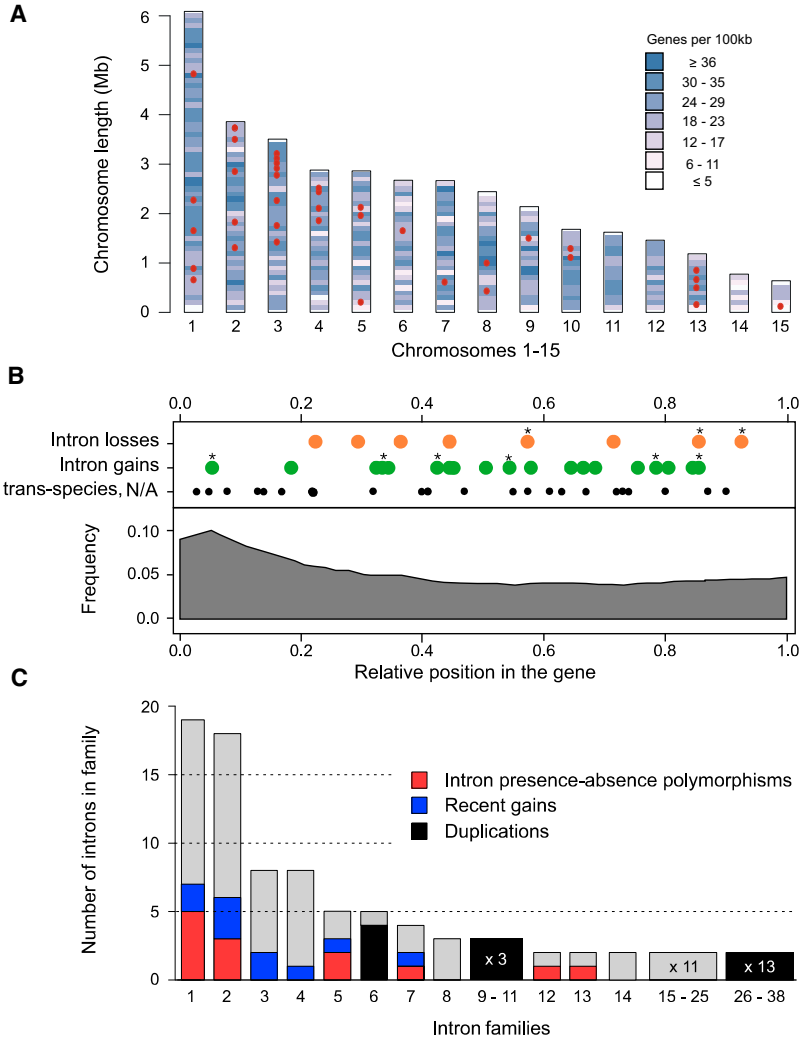

Figure 2. Intron Locations and Clustering of Intron Sequences

(A) Schematic representation of the 15 largest chromosomes of *M. graminicola*. Blue shadings indicate gene density in 100 kb intervals in the reference genome IPO323. Red dots indicate chromosomal locations of the genes displaying intron PAPs in *M. graminicola* (see Table S1 for details on the intron PAP loci). Figure S4 shows data on gene and intron length distributions and gene density in *M. graminicola*.

(B) The relative intron position (0–1) for all introns in fully annotated *M. graminicola* genes showed a 5′ bias (dark gray). The relative positions for all 52 intron PAPs found within *M. graminicola* are shown as circles. Intron losses are orange; intron gains are green. Black dots represent transspecies intron PAPs and cases where the ancestral state could not be determined. Asterisks mark intron gains or losses in genes larger than 2,500 bp. In large genes, intron deletions due to reverse transcription of spliced RNA are expected to increase toward the 3′ terminus of the gene.

(C) Families of highly similar intron sequences in *M. graminicola*. Introns in the same family share more than 80% sequence identity and more than 80% length similarity. Intron PAPs are red. Introns that are fixed in *M. graminicola* but absent in S1 are blue. Introns originating from complete or partial gene duplication are shown in black. See Table S4 for a complete list of members of each intron family.

of many IGL events. Modeling underscored the importance of effective population size in predicting rate variations in IGL, and drastic changes in intron densities during the evolution of multicellular organisms have been proposed to be driven by reductions in effective population size [18].

The frequency of intron PAP at locus ID-54115 was highly variable, ranging from 0% to 90% among *M. graminicola* populations, but was fixed in both S1 and S2 (Figure 1C). This example illustrates a rapid intron loss, because half of the sampled populations completely lacked the intron. Rapid intron gains were observed at loci ID-48846 and ID-60105: both ancestral species lacked the intron, and population frequencies in *M. graminicola* exceeded 75% (Figure 1C; Figure S1; Figure S2). Rapid transitions from intron absence in close ancestors to near fixation in *M. graminicola* may reflect selection acting directly on the intron or on tightly linked loci. Sequence polymorphism in the intron was much lower than in the surrounding exon sequences for locus ID-48846, consistent with a rapid introgression of this intron into these populations. We found a similar pattern of nucleotide diversity at loci ID-72460 and ID-72506 (Figure S3). Intron PAPs at loci ID-84625 and ID-107092 were in transient stages in both *M. graminicola* and S1, illustrating that intron PAPs can persist through speciation and be maintained in multiple populations (Figure 1C; Figure S2). Large effective population sizes in S1 and *M. graminicola* likely contributed to the long-term persistence of these intron PAPs. Nucleotide diversity in the intron and exon sequences was high for locus ID-84625, consistent with long-term persistence of this intron PAP (Figure S3).

Intron Transposition Is a Major Mechanism Generating New Introns

The large number of intraspecific intron PAPs identified among closely related species provides an ideal framework to investigate proximate mechanisms leading to IGLs. From comparative genomics data, we identified 18 gains and 8 losses within *M. graminicola* (14 intron PAPs were transspecific and 12 were ambiguous; Table S1). A major mechanism to explain intron loss is reverse transcription of spliced mRNA followed by homologous recombination of cDNA with the intron-containing gene [2, 12]. Intron loss through insertion of mRNA was clearly demonstrated in *Cryptococcus neoformans* [6]. Intron deletions in *M. graminicola* showed a slight bias toward the 3′ terminus of the gene, consistent with the prediction for this mechanism and evidence from comparative genomics among *Cryptococcus* species (Figure 2B; [7]). In contrast, intron gains in *M. graminicola* were evenly distributed across genes. The insertion of novel introns could preferentially occur in between-codon positions (phase 0), because subsequent intron sliding would be more deleterious for phase 1 or 2 insertions [18]. We found that intron PAPs in *M. graminicola* were biased toward phase 0 insertions (43.2%) compared to phase 1 and 2 (31.8% and 25.0%, respectively). However, genomic averages of intron insertion phases were very similar (39.2%, 33.3%, and 27.5% for phases 0, 1, and 2, respectively).

Intron sequences recently gained through transposition may show a high sequence similarity to other introns (the putative

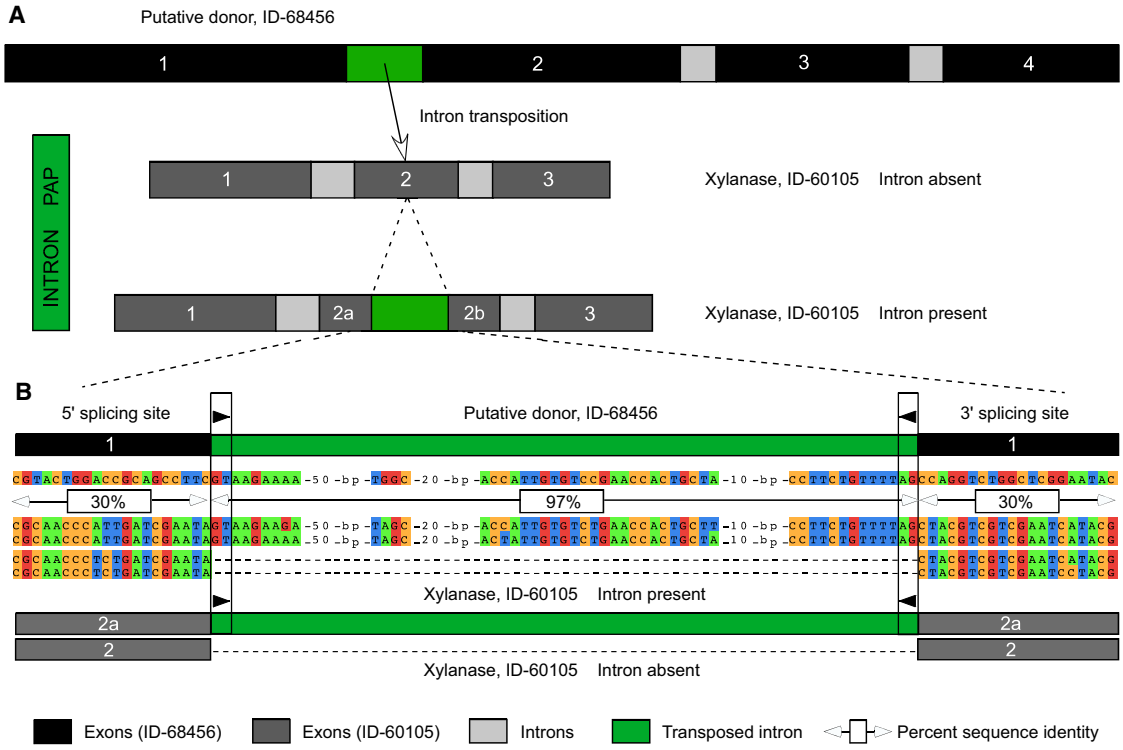

Figure 3. Intron Transposition from a Putative Donor Gene into the Xylanase Gene

(A) Exons are black (ID-68456) or dark gray (ID-60105, xylanase). Introns are light gray except for the transposed intron (green). The transposed intron shows PAP in the xylanase gene.

(B) A five-sequence alignment covering the intron insertion point includes the putative donor ID-68456 and four xylanase sequences with (top) or without (bottom) the intron. Percent sequence identity between the sequences in different regions is indicated in boxes. The transposed intron showed very high (97%) sequence identity, whereas no exon sequence homology was found beyond the splicing sites (black arrows). See Figure S2 for a multiple sequence alignment of the xylanase gene.

donors). Evidence of intron transposition has remained largely elusive [4]. However, several pairs of highly similar introns were recently identified in the pelagic tunicate *Oikopleura* [15]. We screened all *M. graminicola* introns to identify possible families of intron sequences. Thirty-eight families of highly related intron sequences were identified, containing 133 introns (0.77% of all introns; Table S4). We excluded 16 intron families because these likely originated through either partial or complete duplication of the gene containing the intron. The remaining intron families contained 13 introns existing as intron PAPs in *M. graminicola* and 10 recently gained introns that were fixed in *M. graminicola* but absent in S1 (Figure 2C). The high number of intron PAPs and recently gained introns sharing high sequence similarity suggests that intron transposition plays a major role. Similar families of highly related intron sequences were found in S1 and S2 (data not shown).

The intron PAP of locus ID-60105 (xylanase), found in intron family 5, is almost fixed in *M. graminicola* (98% of isolates) but is completely absent in the ancestral species S1, S2, and *S. passerinii* (Figure 1C; Table S3). This very recent transposition allowed us to identify the two most likely intron donor loci (ID-68456 and ID-103686) and localize precisely the position of intron integration (Figure 3). Another intron from family 5, found in locus ID-74664, was polymorphic for presence/absence, representing an intron loss in *M. graminicola*. Two of the eight introns in family 4 were in the same gene (ID-38371). One of these two introns was absent in S1, consistent with a very recent intragenic intron transposition. We found no evidence that genomic context played a role in intron transposition (Figure 2A; Figure S4).

Intron transposition in *M. graminicola* occurred both within transcripts, as shown previously in the tunicate *Oikopleura* [15], and among distant genomic locations. This suggests that a common mechanism underlies the transposition of introns both within the same or among different transcripts. Molecular mechanisms for intron transposition among unrelated genes are unknown except for transposon-related insertions likely requiring specific sequence signals [13]. Transposed introns in *M. graminicola* were short in length (<130 bp) and therefore may be unable to encode a functional transposon machinery. However, miniature inverted-repeat transposable elements (MITEs), an abundant class of transposable elements in plants and animals, were associated to recently gained introns in tunicates [15]. Recently gained introns in *Caenorhabditis elegans* were shown to share palindromic sequences [28]. Several recently gained introns in *M. graminicola* showed evidence for inverted repeats; however, all identified motifs were imperfect and did not directly flank intron splicing sites (data not shown). Alternatively, introns may be transposed through reverse splicing involving an armed spliceosome carrying a recently spliced intron [13, 29]. The large families of highly similar intron sequences found in these genomes suggest that certain intron sequences are much more likely to be transposed than others and that specific sequence patterns may promote transposition.

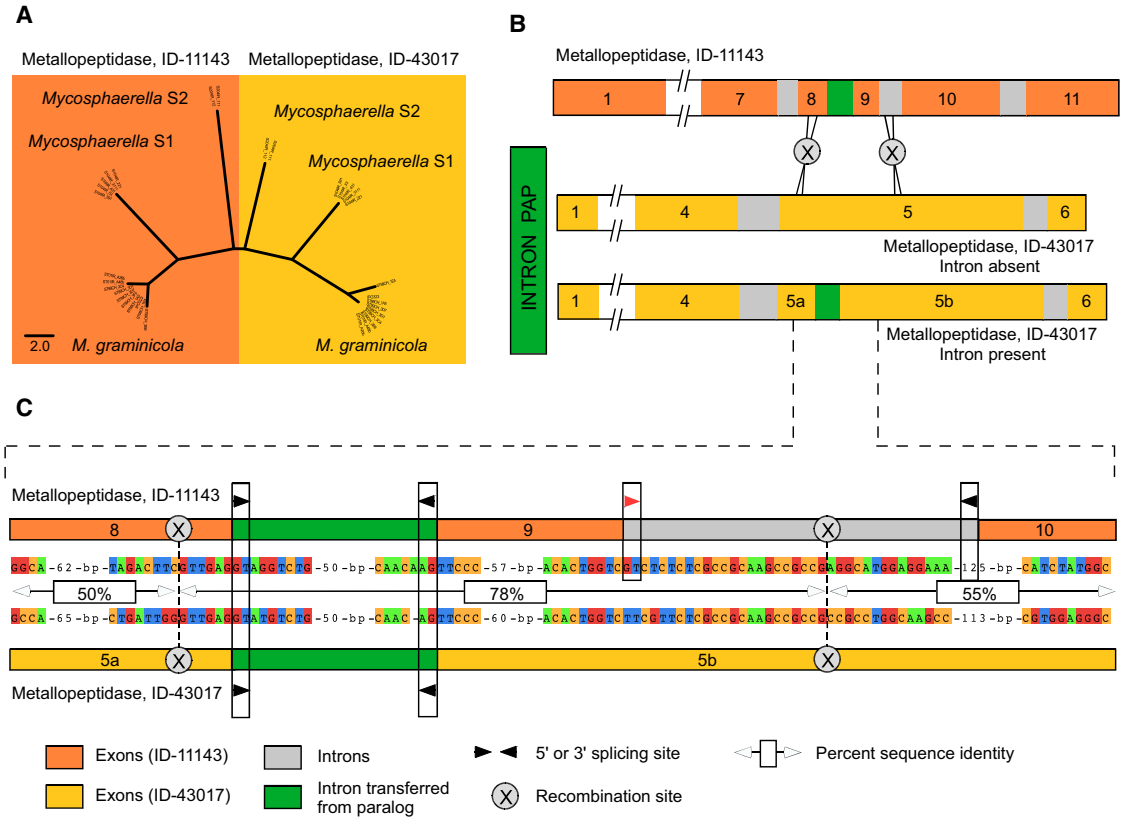

Figure 4. Intron Transfer between Two Metallopeptidase Paralogs

(A) Unrooted neighbor-joining tree of intron sequences found at the paralogous intron position in two metallopeptidases (ID-11143 and ID-43017). S2 intron sequences were the most closely related to each other, with a gradual divergence among the intron sequences in S1 and *M. graminicola.*

(B) Exons are shown in orange (ID-11143) or yellow (ID-43017). Introns are light gray, except for the transferred intron, shown in green. The transferred intron showed PAP in S2. The most likely mechanism for intron transfer was a double recombination event between the two metallopeptidase paralogs.

(C) Sequence alignment of the two metallopeptidase paralog sequences from S2. The alignment includes the transferred intron (green) and the flanking regions including the most likely recombination breakpoints (gray circles). The 5′ recombination site was found in the exon for both paralogs, but the 3′ recombination site was found in an intron of ID-11143 and in an exon of paralog ID-43017. The mutated 5′ splicing site from GT to TT is marked with a red arrow.

Intron family 14 (Figure 2C) contained two highly related introns from two paralogous metallopeptidases encoded by ID-11143 and ID-43017 located on chromosomes 2 and 5 (Figures 4A and 4B). In S2, the corresponding intron of *M. graminicola* metallopeptidase ID-43017 displayed intron PAP (Table S2). Paralogous introns in S2 showed higher similarity than paralogous introns in S1 and *M. graminicola* (Figure 4A). This pattern indicates that the intron transfer among paralogs probably occurred in S2 or in an unknown ancestor because metallopeptidase ID-43017 lacked the intron in *S. passerinii* (Table S3). Using sequence alignments, we showed that the highest sequence similarity extended to the coding sequences immediately adjacent to the introns (Figure 4C). This finding shows that intron gains can result from recombination in the intron-flanking regions among paralogs (Figure 4C). Intron transfer among paralogs differs from intron transposition in that it requires sequence similarity among donor and recipient genes. We identified only a single case of intron transfer among paralogs in the clade of *M. graminicola*. However, the analyzed genomes possess few recently duplicated genes [25], and we expect that a substantially larger number of intron transfers among paralogs may be found in genomes with a higher proportion of recent gene duplicates.

A previous study analyzing distantly related fungi revealed that some genes were hotspots for IGLs over evolutionary time [30]. Five genes in *M. graminicola* showed multiple intron PAPs (Table S1). To our knowledge, this is the first report of multiple intron PAPs occurring simultaneously within a single gene of a species. For all five genes, the most parsimonious explanation for multiple intron PAPs within a gene is the retention of ancestral sequences within the locus. Incomplete lineage sorting is frequent within the studied fungal clade [26] and provides a previously unrecognized mechanism for the retention of intron PAPs within species. This contrasts with multiple independent intron gains at the same intron position that were reported in *Daphnia* [5].

Conclusions

By combining comparative and population genomics with population genetics, we were able to identify a large number of the crucial transient stages of intron PAPs and differentiate proximate mechanisms that generate IGLs. We present direct evidence of gains through intron transposition and intron transfer among paralogs. The discovery of families of highly related intron sequences provides a powerful genomic signature to screen for recent IGLs in other organisms. Intron PAPs were identified in all three species using population

genomic data, suggesting that transient stages in the gain or loss of introns may be much more common than previously thought from the comparison of evolutionarily distant genomes.

Supplemental Information

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.10.041.

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